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Deletion of *WNK1* First Intron Results in Misregulation of Both Isoforms in Renal and Extrarenal Tissues

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Abstract—Large deletions in intron 1 of the with-no-lysine kinase type 1 (*WNK1*) gene cause familial hyperkalemic hypertension. Alternative promoters generate functionally different isoforms: long ubiquitous isoforms (L-WNK1) and a kidney-specific isoform (KS-WNK1) lacking kinase activity. It remains unclear whether the disease-causing mutations selectively modify the synthesis of 1 or both types of isoforms. Using a transgenic mouse model, we found that intron 1 deletion resulted in the overexpression of *L-* and *KS-WNK1* in the distal convoluted tubule and ubiquitous ectopic *KS-WNK1* expression. Phylogenetic and functional analysis of the minimal 22-kb intron 1 deletion identified 1 repressor and 1 insulator, potentially preventing interactions between the regulatory elements of L-WNK1 and KS-WNK1. These results provide the first insight into the molecular mechanisms of WNK1-induced familial hyperkalemic hypertension. **(*Hypertension*. 2008;52:1149-1154.)**

Key Words: hypertension ■ WNK1 ■ FHHt ■ transgenic ■ insulator ■ repressor

Mutations in the *WNK1* and *WNK4* genes, encoding 2 members of the with-no-lysine (WNK) subfamily of serine threonine kinases, are responsible for familial hyperkalemic hypertension (FHHt), a rare form of human arterial hypertension, also known as pseudohypoaldosteronism type 2 or Gordon syndrome.[1] In vivo and in vitro experiments have shown this new regulatory pathway to be important for ion handling and blood pressure regulation.[2] Two functionally different WNK1 isoforms have been characterized: ubiquitous, full-length, catalytically active isoforms (L-WNK1) and a short kidney-specific isoform (KS-WNK1), catalytically inactive and present only in the renal distal tubule.[3] The balance between L-WNK1 and KS-WNK1 seems crucial for sodium and potassium handling regulation.[4]

The molecular mechanisms controlling *WNK1* expression are only partly understood. In humans, 2 proximal promoters (pP) control *L-WNK1* transcription, and a third renal promoter (rP), in intron 4 and 98 kb away from pP, controls *KS-WNK1* expression.[5] The large overlapping deletions (41 kb and 22 kb) within intron 1 (i1) of *WNK1* observed in FHHt kindreds highlight the importance of intron 1 in regulating *WNK1* expression. *WNK1* mRNA levels in leukocytes are ≈5 times higher in affected individuals than in controls.[1] It remains unclear whether intron 1 deletions selectively modify the expression of 1 or both isoforms and the tissue specificity of these changes.

We have shown that the human and mouse *WNK1* genes are similarly organized and expressed.[5] We have also demonstrated the feasibility of a transgenic approach based on bacterial artificial chromosomes (BACs) for the detailed analysis of *WNK1* expression.[6] Here, we created a new BAC transgene for the qualitative and quantitative analyses of the pattern of *L-WNK1* and *KS-WNK1* expression in the presence and absence of intron 1. We also carried out a comparative, interspecies sequence analysis, together with in vitro functional analysis, and identified a repressor and an insulator potentially responsible for the misregulation of WNK1 isoforms.

Methods

Generation of the Transgene and Embryonic Stem Cell Clones

BAC RP24-212e14, spanning the *mWNK1* locus, has been described elsewhere.[6] This BAC was modified using the same techniques. LoxP and LoxP511 sites of the BAC backbone were replaced with a *pgk-EM7-hygro* and a *pgk-puro* selection cassette, respectively, flanked by recognition sites for the ϕC31 integrase to allow their deletion in embryonic stem (ES) cells.[7] The *nlacZ* gene, encoding a nuclear β-galactosidase (β-gal), was inserted at the ATG of exon 4a. An *IRES-luc* (internal ribosome entry site-luciferase) cassette was inserted into exon 2. LoxP2272 sites were inserted 47 bp downstream from exon 1 and 95 bp upstream from exon 2.

BAC DNA was linearized with *PI-SceI*, purified, and checked by pulse-field gel electrophoresis. CK35 ES cells[8] were electroporated

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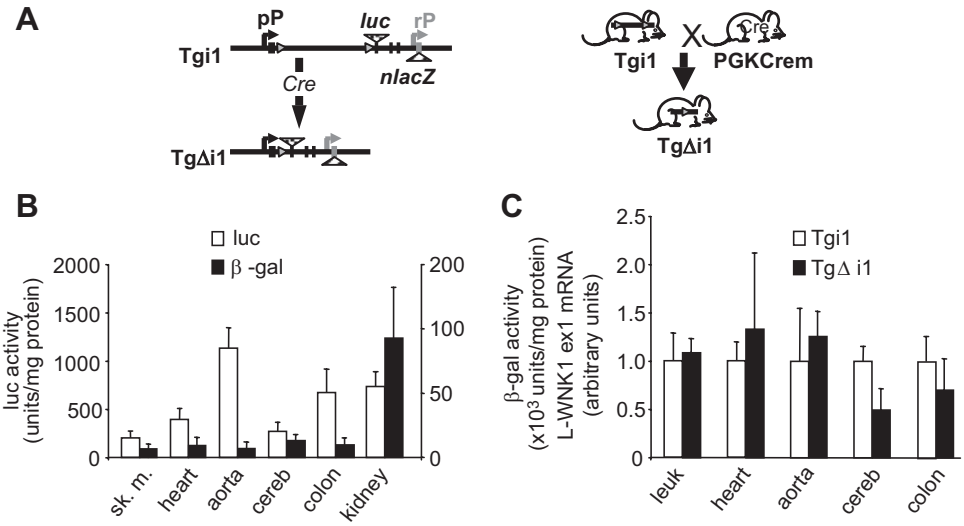

Figure 1. Extrarenal expression of *L-WNK1* is unaffected by intron 1 deletion. A, Generation of Tgi1 and TgΔi1 animals. Tgi1 transgene carries the entire intron 1 of *WNK1*. Cre-mediated recombination leads to deletion of intron 1 in TgΔi1 animals, obtained by breeding the Tgi1 animals with PGKCrem mice. B, Transgene expression is similar to endogenous *L-WNK1* and *KS-WNK1* expression, as determined by quantification of luciferase (□) and β-gal (■) activity, respectively. C, Comparison of the expression level of *L-WNK1* exon 1 in mRNA extracts from Tgi1 and TgΔi1 adult mouse tissues (n=4 to 5). Results are expressed as means+SEMs. Sk. m indicates: skeletal muscle; cereb, cerebellum; leuk, leukocytes.

with 10 μg of linearized BAC DNA using a BTX ECM 830 pulse generator. Puromycin (2 μg/mL) and hygromycin (200 μg/mL) were added for selection 2 and 3 days after electroporation, respectively. Genomic DNA (40 μg) was digested with *Pst*I for Southern blotting analysis. The sequences of exon 2 and exon 4a were used as a probe. The hybridization signal was quantified with a Molecular Imager FX and Quantity One software (Bio-Rad). Luc and β-gal activities were measured with the Dual-Light Luminescent Reporter Gene Assay (Applied Biosystems), using a Glomax luminometer (Promega), and normalized according to the protein concentration measured with the bicinchoninic acid protein assay (BCA kit, Sigma).

Generation and Identification of Tgi1 and TgΔi1 Transgenic Mice

All of the experimental procedures were performed in accordance with the French government animal welfare policy. Tgi1 mice were generated by microinjecting ES cells into blastocysts, using standard techniques.[9] Chimeric founders were crossed with C57BL/6N animals to establish the Tgi1 line. Tgi1 males were crossed with PGKCrem females to establish the TgΔi1 line. All of the animals were genotyped by PCR. Primer sequences are given in the online data supplement (Table S1, available at http://hyper.ahajournals.org).

Analysis of Transgene Expression

X-gal staining and real-time RT-PCR on microdissected tubules were performed as described.[6] Luc and β-gal activities were determined in protein extracts of adult tissues, as described for ES cells. The sequences of the primers used to amplify WNK1 exon 1 are given in Table S1. All of the data are presented as means+SEMs. Comparisons among groups were assessed by unpaired *t* test. Differences were considered significant at $P \leq 0.05$.

Transient Transfection and Luciferase Assays

Plasmids P1–200, P2+626, and rP-5269 have been described elsewhere.[5] C1–5 and fragments F1 to F10 were cloned by PCR and inserted in the sense and antisense orientations into the *Sal*I site of the pGL3-promoter vector (Promega), P1–200, P2+626, or rP-5269 to assess their potential repressor activity. C1–5 was inserted into the *Sal*I and/or *BsmBI* sites of the rP-5269 plasmid to test for insulator activity. Two copies of C5 were also inserted in the *Sal*I and *Not*I sites to create rP-C5C5. Cell cultures, transient transfections, and reporter gene assays were carried out as described.[5]

Results

Generation of the Transgenic Reporter Model

We used the Cre-loxP system to delete *WNK1* intron 1 from the complete BAC transgene after its insertion into the mouse genome to prevent possible discrepancies in the expression pattern because of differences in the integration site. The detailed strategy that we used to generate the Tgi1 transgene, the corresponding ES cell clones, and the mouse line is described in Figure S1. Briefly, *luc* and *nlacZ* reporter genes were inserted into *mWNK1* exon 2 and at the ATG of exon 4a to monitor *L-WNK1* or *KS-WNK1* expression, respectively. LoxP2272 sites were inserted at the 5′ and 3′ ends of intron 1. Transgenic Tgi1 mouse chimeras were generated by injection of the selected ES clone into blastocysts, and TgΔi1 mice were obtained by crossing Tgi1 males with PGKCrem females[10] (Figure 1A).

Consequences of the Deletion of WNK1 Intron 1 in Extrarenal Tissues

We first verified that the pattern of expression of luc and β-gal in Tgi1 mice was similar to that of *WNK1*. Like *L-WNK1*, luc activity was ubiquitously detected (Figure 1B). Significant β-gal activity was detected only in the kidney (Figure 1B), and quantitative real-time PCR on microdissected tubules showed that *nlacZ* transcripts are highly expressed in the distal convoluted tubule (DCT) and connecting tubule (see below), consistent with the pattern of endogenous *KS-WNK1* expression.

Before analyzing the consequences of the deletion of intron 1 on reporter gene expression, we also measured the level of expression of the 1-copy transgene compared with endogenous *WNK1*. Real-time PCR on kidney extracts showed that the *luciferase* gene was 100 times less expressed than the endogenous gene despite the large regions included in the BAC upstream and downstream of the *WNK1* gene (Figure

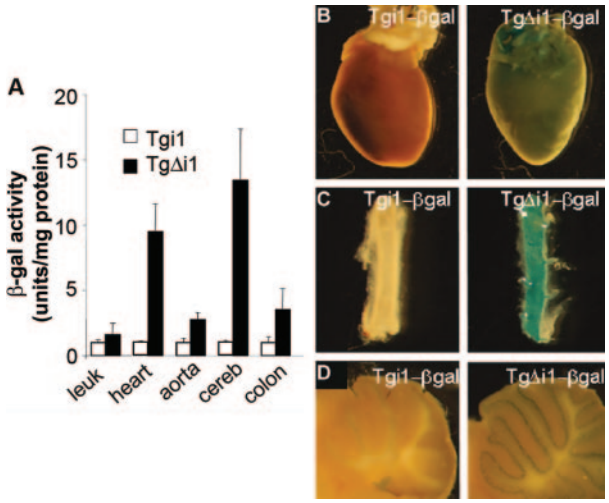

Figure 2. Ectopic expression of β-gal in TgΔi1 animals. A, Comparison of β-gal activity in protein extracts from Tgi1 and TgΔi1 adult mouse tissues (n=3 to 14). β-Gal activity levels in all of the tissues of Tgi1 mice were arbitrarily set at 1. Activity was normalized according to total protein concentration. Results are expressed as means+SEMs: β-Gal activity is significantly increased in all of the tissues ($P<0.02$) except the colon. B through D, Whole-mount X-gal staining of Tgi1 tissues (left) and TgΔi1 tissues (right). Ectopic expression is observed in the heart (B), aorta (C), and cerebellum (D).

S2). Inappropriate splicing of a large internal exon within a transgene having been reported, we investigated the possible skipping of the modified *WNK1* exon 2.[11] RT-PCR between *L-WNK1* exon 1 and IRES showed that correct splicing occurred between exon 1 and the IRES-luc cassette. However, RT-PCR between exons 1 and 3 revealed that skipping of the modified exon 2 did take place in Tgi1 tissues (Figure S2). The same event was seen in TgΔi1 tissues, but to a much lesser extent. This result means that potential modification of luc activity after deletion of *WNK1* intron 1 would not reflect the sole effect of this deletion but also of a modified level of ex2-IRESluc skipping. We, therefore, measured the variation in transgenic *L-WNK1* expression after intron 1 deletion by the quantification of exon 1 expression by real time RT-PCR. This technique allowed us to show that deletion of the *WNK1* first intron did not modify *L-WNK1* expression in extrarenal tissues (Figure 1C).

We then investigated the effects of intron 1 deletion on KS-WNK1 reporter expression. Significant levels of β-gal activity were detected in all of the extrarenal tissues of TgΔi1 mice, whereas only background levels were detected in Tgi1 mice (Figure 2A). This ectopic β-gal activity was particularly strong in skeletal muscle, heart, and cerebellum, in which β-gal activity was ≈1.6 to 13.0 times stronger than background levels. In accordance, X-gal staining was observed in the heart (Figure 2B), the vascular tree (Figure 2C), and cerebellar Purkinje cells (Figure 2D). Interestingly, these tissues correspond with those with strong endogenous *L-WNK1* expression.[6] Ectopic β-gal expression was also seen in leukocytes, whereas no overexpression of *L-WNK1* was detected (Figures 1C and 2A). This result suggests that the overexpression of *WNK1* seen in the leukocytes of FHHt patients is attributable solely to the ectopic expression of *KS-WNK1*. This was confirmed by RT-PCR with primers specific for the short isoform (data not shown).

Consequences of Intron 1 Deletion on Renal WNK1 Expression

We first showed that exon 1 expression is unchanged in extracts prepared from the whole kidney of Tgi1 and TgΔi1 animals, whereas β-gal activity was 4 times stronger in TgΔi1 kidneys ($P=0.02$; Figure 3A). We then used real-time RT-PCR on microdissected tubules to investigate the consequences of intron 1 deletion in each nephron segment. Exon 1 expression was not modified throughout all of the nephron segments of TgΔi1 except the DCT, in which expression level was 3 times higher than in Tgi1 mice ($P=0.03$; Figure 3B). This pattern of expression in TgΔi1 kidneys, therefore, resembles that of endogenous *KS-WNK1*. As expected, *nlacZ* expression was strong in the DCT of Tgi1 mice (Figure 3C). TgΔi1 mice displayed 4 times higher levels of *nlacZ* expression in this segment ($P<0.05$; Figure 3C). A smaller increase was also observed in other nephron segments but was difficult to interpret, because it was close to the background signal (data not shown).

Taken together, the analysis of transgene expression in renal and extrarenal tissues suggests that 2 types of regulatory elements could be present within the *WNK1* first intron. First, 1 or several repressor elements could constitutively repress *KS-WNK1* expression in all of the extrarenal tissues and the expression of both isoforms in the DCT. Second, an insulator could prevent illegitimate interactions between *L-WNK1* regulatory elements and rP. Deletion of this element could lead

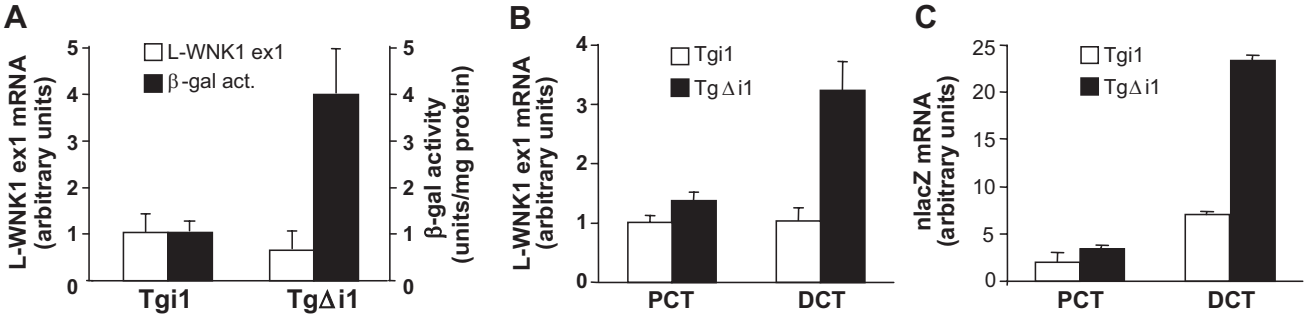

Figure 3. Comparison of transgene expression in the kidneys of Tgi1 and TgΔi1 mice. A, Comparison of *L-WNK1* exon 1 mRNA expression level (□) and β-gal activity (■) in kidneys from Tgi1 and TgΔi1 adults. B and C, Relative abundance of *L-WNK1* exon 1 (B) and *nlacZ* (C) mRNA along the dissected mouse nephron, as determined by real-time RT-PCR. Experiments were performed on the proximal convoluted tubule (PCT) and DCT. Results are expressed as means+SEMs from 3 to 4 animals.

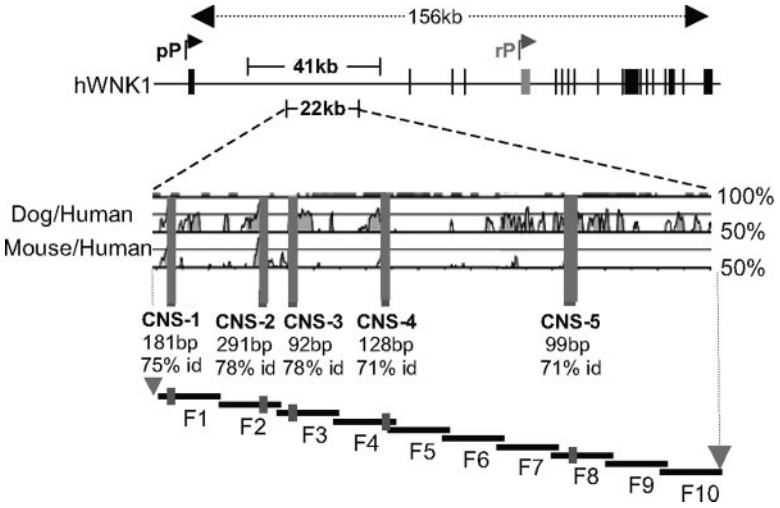

Figure 4. Cross-species comparison of the 22-kb sequence in *WNK1* intron 1 deleted in FHHt. VISTA Genome Browser output in which the human sequence is used as the reference sequence, with percentage of similarity to dog or mouse sequences plotted on the vertical axis. Five CNSs (▨) were identified. The position of the core sequence relative to the first nucleotide of the human intron 1, its length, and the percentage sequence identity (% id) between the mouse and human sequences are indicated. The human 22-kb sequence was truncated into 10 overlapping fragments designated F1 to F10.

to ubiquitous expression of *KS-WNK1* and overexpression of *L-WNK1* and *KS-WNK1* in the DCT.

Several Repressors in *WNK1* Intron 1

We used a systematic approach to search for an intronic repressor. The transcriptional activity of ten 2.5-kb overlapping fragments covering the minimal 22-kb intron sequence deleted in FHHt patients was tested in vitro (F1 to F10, Figure 4, and Table S2). Because our in vivo model suggested that intron 1 contained a repressor affecting the activity of both pP and rP, we used the heterologous SV40 promoter (pSV40) to control *luc* expression. Transient luciferase assays were carried out in renal human embryonic kidney, Madin-Darby canine kidney, and Chinese hamster ovary cells. The more distal fragment, F1, repressed pSV40 when inserted in sense or antisense orientation, resulting in a decrease in transcriptional activity by a factor of 2 to 14. To a lesser extent (25% to 50%), F7 repressed pSV40 in all 3 of the cell lines. F3 displayed strong repressor activity in human embryonic kidney and Chinese hamster ovary cells only. These analyses suggest that there are several repressor sequences within intron 1, with the major and most reproducible transcriptional effect being because of F1.

Five Potential Regulatory Elements Identified by Cross-Species Sequence Comparison

We also searched for the corresponding potential regulatory elements (repressor and/or insulator) by carrying out a database search using VISTA (http://www.gsd.lbl.gov/vista). Regulatory elements are usually composed of highly conserved (>70% identity) long sequences (>100 bp long).[12] We identified 6 conserved noncoding sequences (CNSs, or Cs) among the human (gi: 89035948), mouse (gi: 94378178), and dog (gi: 54126074) genomes that met these criteria. The order of the 6 blocks is conserved among the 3 species. C1 through C5 are located within the minimal 22-kb human deletion responsible for FHHt (Figure 4). CNS6 is also excluded from the 41-kb *WNK1* intron 1 deletion observed in the other FHHt kindred. The core sequences of the CNSs display a high degree of sequence similarity, exceeding 71% in mice and humans.

One of the CNSs Acts as a Repressor of rP In Vitro

We assessed the transcriptional activity of the 5 CNSs by subcloning each sequence downstream from the *luc* gene under the control of pSV40, pP, or rP. The resulting constructs were used to transfect renal human embryonic kidney, Madin-Darby canine kidney, and Chinese hamster ovary cells to evaluate the cell specificity of the CNS activity (data not shown). C1 was the only one that displayed repressor activity. C1 decreased rP promoter activity in Madin-Darby canine kidney cells by a factor of 2 to 3 (Figure 5A). This repressor effect was partly dependent on orientation, because placing C1 in the antisense orientation decreased rP activity by 50%

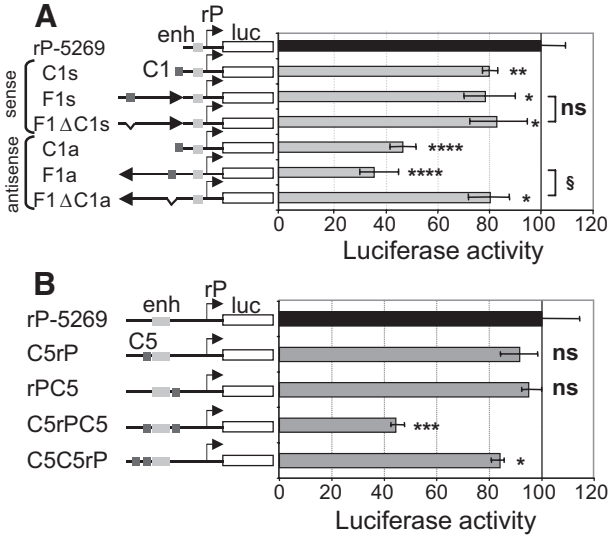

Figure 5. Functional analysis of the CNS in Madin-Darby canine kidney (MDCK) cells. A, The transcriptional effect of F1 (arrow), C1 (■), and C1 deleted of F1 (F1ΔC1) in the sense (s; 5′-3′) or antisense (a; 3′-5′) on rP activity was tested by a luciferase transient assay. B, Enhancer-blocking activity of C5. One copy of C5 (■) was inserted on each side of the renal enhancer (enh) or between enh and rP. In addition, 2 copies of C5 were also inserted between enh and rP as a control. Luciferase activity was normalized with respect to that of rP-5269. Histograms represent means±minimums and maximums for 3 experiments. ns indicates nonsignificant. *$P<0.05$; **$P<5\times10^{-4}$; ***$P<5\times10^{-5}$; ****$P<1\times10^{-9}$ vs rp-5269; §$P<1\times10^{-4}$ vs F1a.

to 60%, whereas, in the sense orientation, C1 decreased rP activity by only 20% to 30%. C1 did not repress pSV40 or pP activity. In silico analysis of C1 revealed that it contains several consensus sequences for known repressors (Figure S3). C1 may, therefore, be responsible for the overexpression in the DCT of *nlacZ* (KS-WNK1) observed in TgΔi1 mice.

We then investigated whether C1 was responsible for the repressor effect of F1. The repressor activity of F1 on rP was found to be similar to that of C1. F1 repressed rP activity by 50% to 60% in the antisense orientation but only by 20% to 30% in the sense orientation (Figure 5A). The deletion of C1 from F1 significantly decreased the repressor effect of F1: F1ΔC1 in the antisense orientation repressed rP activity by only 30% (Figure 5A). This effect was less significant in the sense orientation. These results confirm the repressor effect of C1 in a larger genomic context but suggest that F1 contains other repressor sequences.

Identification of an Insulator-Like Element In Vitro

We then investigated whether one of the CNSs could act as an insulator, blocking the activity of the renal enhancer, located in intron 4a.[5] C1 through C5 were placed upstream from, downstream from, or on either side of the renal enhancer. Only C5 had an effect compatible with an insulator sequence (Figure 5B). A single copy of C5 did not decrease rP transcriptional activity. However, surrounding the enhancer with 2 copies of C5 decreased rP transcriptional activity by 55%, suggesting that the enhancer was no longer able to activate rP. No such decrease was observed if 2 copies of C5 were inserted downstream from the enhancer, so the decrease in transcriptional activity was because of enhancer-blocking activity rather than an additive effect of multiple copies of C5. The insulator effect of C5 was also seen on the SV40 promoter (Figure S4). C5 may, therefore, block the interaction between the renal enhancer and pP. Interestingly, C5 contains 4 consensus sequences for the CCCTC-binding Factor CTCF (Figure S3), the only identified transacting factor that confers enhancer-blocking insulator activity.[13]

Model for Intron 1 Function in WNK1 Transcriptional Regulation

Our in vivo and in vitro results suggest that *WNK1* intron 1 contains at least several constitutive repressors acting on pP and rP to repress the expression of *L-WNK1* and *KS-WNK1* and/or one or several insulators limiting interactions between the regulatory elements of *L-WNK1* and *KS-WNK1*. We can, thus, propose a model for the regulation of WNK1 isoform transcription by intron 1, accounting for the consequences of its deletion in patients with FHHt (Figure 6). Deletion of the repressor sequences leads to the overexpression of *L-WNK1* and *KS-WNK1* in the DCT (Figure 6A). Deletion of the insulator leads to illegitimate interactions between regulatory modules controlling *L-WNK1* and *KS-WNK*1 expression, resulting in *L-WNK1* overexpression in the DCT and *KS-WNK1* ubiquitous expression (Figure 6B).

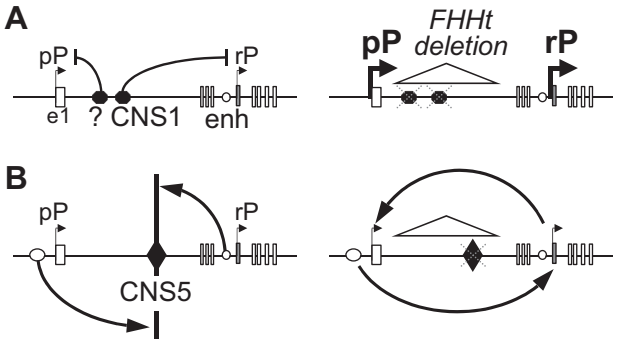

Figure 6. Model of *WNK1* normal and pathological transcriptional regulation. A, *L-WNK1* and *KS-WNK1* physiological expressions are constitutively repressed by ≥2 distinct repressors (black hexagons) located in the first intron of the gene. C1 could repress rP activity. Deletion of the repressor leads to *KS-WNK1* overexpression in the DCT. B, An insulator (black rhombus) prevents illegitimate interactions between L-WNK1 and KS-WNK1 regulatory elements (white ovals). This element could be C5. Its deletion leads to *L-WNK1* overexpression in the DCT and *KS-WNK1* ectopic expression in extrarenal tissues.

Discussion

Elucidation of the regulatory pathways involving the intron 1 of *WNK1* is important to improve our understanding of the control of normal gene expression and of the way in which it is compromised in FHHt patients. Our transgenic model provides the first insight into the molecular mechanisms of FHHt mutations. Using an in vivo model and a combination of comparative genomic and functional analysis, we characterized the consequences of the deletion of *WNK1* intron 1 on the expression of its isoforms and identified several intronic repressors and insulators.

In the DCT in particular, the deletion of *WNK1* intron 1 leads to increased L-WNK1 and KS-WNK1 expression. Quantification of L-WNK1 exon 1 by real-time PCR on microdissected tubules showed that exon 1 expression is multiplied by 3 in the DCT after deletion of intron 1. This overexpression is consistent with the suspected role of L-WNK1 in the development of hyperkaliemic hypertension. In vitro studies have, indeed, suggested that L-WNK1 prevents the WNK4-mediated inhibition of NCC[14] and inhibits ROMK activity.[15,16] The increase in L-WNK1 level over WNK4 and the relative increase of L-WNK1 level over KS-WNK1 induced by FHHt deletions may, therefore, prevent the Na-Cl cotransporter inhibition of the NCC by WNK4 and accentuate the inhibition of the Renal Outer Medullary potassium channel ROMK. Moreover, L-WNK1 has been shown to phosphorylate SGK-1, leading to the activation of the Epithelial sodium Channel EnaC, independent of WNK4 and KS-WNK1.[17] An increase of L-WNK1 expression in the DCT could, therefore, lead to an increased activation of this channel, accentuating the hypervolemia because of increased sodium absorption by NCC.

Analysis of our transgenic model provides the first evidence that sequences in intron 1 may repress the transcription of *L-WNK1* and *KS-WNK1* and prevent illegitimate interactions between *WNK1* isoforms regulatory elements. A comparative analysis of intron 1 sequences of the human, mouse, and dog *WNK1* genes identified 6 CNSs, 5 of which were located within the FHHt deletions. One of these elements, C1, is located within a 2.5-kb fragment (F1) that decreased reporter gene expression to 40% to 65% of control levels,

without promoter specificity. C1 contains some of the elements required for this repression. C5 acted as an insulator in vitro, blocking renal enhancer activity. No functional activity was found for C2, C3, and C4. However, these sequences may represent regulatory elements, the activity of which is impaired by isolation from their normal genomic environment. It is also possible that some or all of the CNSs act in cooperation to regulate *WNK1* expression.

Intron 1 of *WNK1* is 60 kb long in humans and 30 kb long in mice. Two large overlapping fragments (22 and 41 kb) were deleted in 2 FHHt families.[1] The intron 1 deletion that we generated in this study was, therefore, not identical to that found in humans. However, 5 of the 6 CNSs identified by comparative sequence analysis were found within the sequences corresponding with the human deletions. The increase in β-gal activity in the leukocytes of TgΔi1 paralleled the increased *WNK1* expression in the leukocytes of FHHt patients, detected by real-time PCR with primers amplifying both L-WNK1 and KS-WNK1 but not with primers specific to L-WNK1.[1,18] Moreover, an 8-kb deletion in the 5′ portion of intron 1 that did not overlap with FHHt deletions was identified in control subjects (estimated allelic frequency: 10%),[1] indicating that this sequence is unlikely to play a major role in controlling *WNK1* expression. These results suggest that the consequences of deleting the entire first intron of the mouse gene are probably similar to those of FHHt deletions.

Perspectives

Several pathophysiological hypotheses have been proposed for FHHt, all consistent with a major role for the kidney, and the distal tubule in particular.[2,3] We can only speculate about the consequences of the ectopic expression of *KS-WNK1*. This isoform has been described as a dominant-negative form of L-WNK1. In vitro and in vivo studies showed that WNK4 can regulate ENaC activity in the colon,[19] where *L-WNK1* is expressed. *L-WNK1* is also expressed in the cardiovascular system during development and adulthood.[6] This expression may account for the early death of *WNK1*$^{-/-}$ embryos and the low blood pressure observed in *WNK1*$^{-/+}$ adults in the absence of metabolic disorders.[20] It will, therefore, be important to determine the contribution of the ectopic expression of KS-WNK1 in the colon and cardiovascular system to sodium reabsorption and blood pressure regulation in FHHt patients.

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Disclosures

None.

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Online Supplements
2 tables and 4 Figures

Deletion of *WNK1* first intron results in misregulation of both isoforms in renal and extrarenal tissues

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Primer name	Location	Sequence
	Tgi1 genotyping	
mE4abg-s	5' exon 4a	CAAAGTCAAGGAGGCAGAGC
mWNK1-3'4a-as	3' exon 4a	GAAAAGCATACTTCCTCAAACAGAA
lac95-as	nlacZ	GTTTTCCCAGTCACGACGTT
	TgΔi1 genotyping	
414endex1-m-s	L-WNK1 exon 1	ACCTCACAGAGAGGAAACTTTGA
ex2bg-a	L-WNK1 exon 2	TTCTTTGAATCTCTGCCTTTCAG
	L-WNK1 exon 1 real-time RT-PCR	
mE1-for	L-WNK1 exon 1	CGCTTTCTCAAATTTGACATC
mE1-rev	L-WNK1 exon 1	AATTCACACCAGGCGACTTC
	Luciferase real-time RT-PCR	
luc-qPCR-s	luciferase	ACAATTGCTTTTACAGATGCACATA
luc-qPCR-as	luciferase	GTATTCAGCCCATATCGTTTCATAG

Table S1. Sequence of the primers used to genotype Tgi1 and TgΔi1 mice and to amplify the *L-WNK1* exon 1 in real-time RT-PCR assays.

		Relative luciferase activity					
		HEK293		MDCK		CHO	
	Orientation	Sense	Antisense	Sense	Antisense	Sense	Antisense
Construct	Position						
pSV40		100	-	100	-	100	-
pSV40-F1	22049-24386	**37 (27,42)**	**7 (6,9)**	**67 (63,72)**	**24 (17,27)**	**51 (44,61)**	**9 (7,9)**
pSV40-F2	24333-26584	14 (10,20)	75 (65,95)	46 (42,51)	105 (98,114)	14 (13,16)	78 (68,87)
pSV40-F3	26523-28977	**7 (6,7)**	**4 (4,5)**	**88 (69,100)**	**44 (33,51)**	**5 (4,6)**	**5 (4,8)**
pSV40-F4	28868-31385	55 (49,64)	95 (66,113)	45 (39,53)	60 (55,66)	53 (38,67)	103 (100,108)
pSV40-F5	31270-33804	149 (137,169)	123 (104,150)	142 (109,169)	85 (76,100)	120 (113,127)	111 (98,123)
pSV40-F6	33716-36093	68 (57,76)	146 (95,211)	88 (74,115)	64 (52,77)	144 (126,169)	157 (135,185)
pSV40-F7	36009-35587	**31 (26,34)**	**19 (19,20)**	**58 (41,75)**	**51 (42,63)**	**60 (45,82)**	**52 (46,56)**
pSV40-F8	38417-40954	150 (98,200)	-	95 (84,101)	-	77 (75,81)	-
pSV40-F9	40865-42568	159 (143,184)	175 (137,239)	96 (78,123)	77 (65,87)	134 (124,146)	160 (151,169)
pSV40-F10	42519-44185	80 (75,95)	125 (113,132)	119 (110,129)	116 (77,147)	81 (71,95)	151 (124,178)

Table S2. Functional analysis of 2.5 kb fragments covering the 22 kb FHHt deletion. Intronic fragments (F) were cloned in the sense or antisense orientation in pGL3-Promoter. The positions are given relative to the first nucleotide of the human intron 1 sequence Reporter plasmids were used to transfect HEK293, MDCK and CHO cells. The results presented are the mean luciferase activity of pSV40-F constructs normalized to that of pGL3-Promoter (minimum, maximum) from at least three experiments.

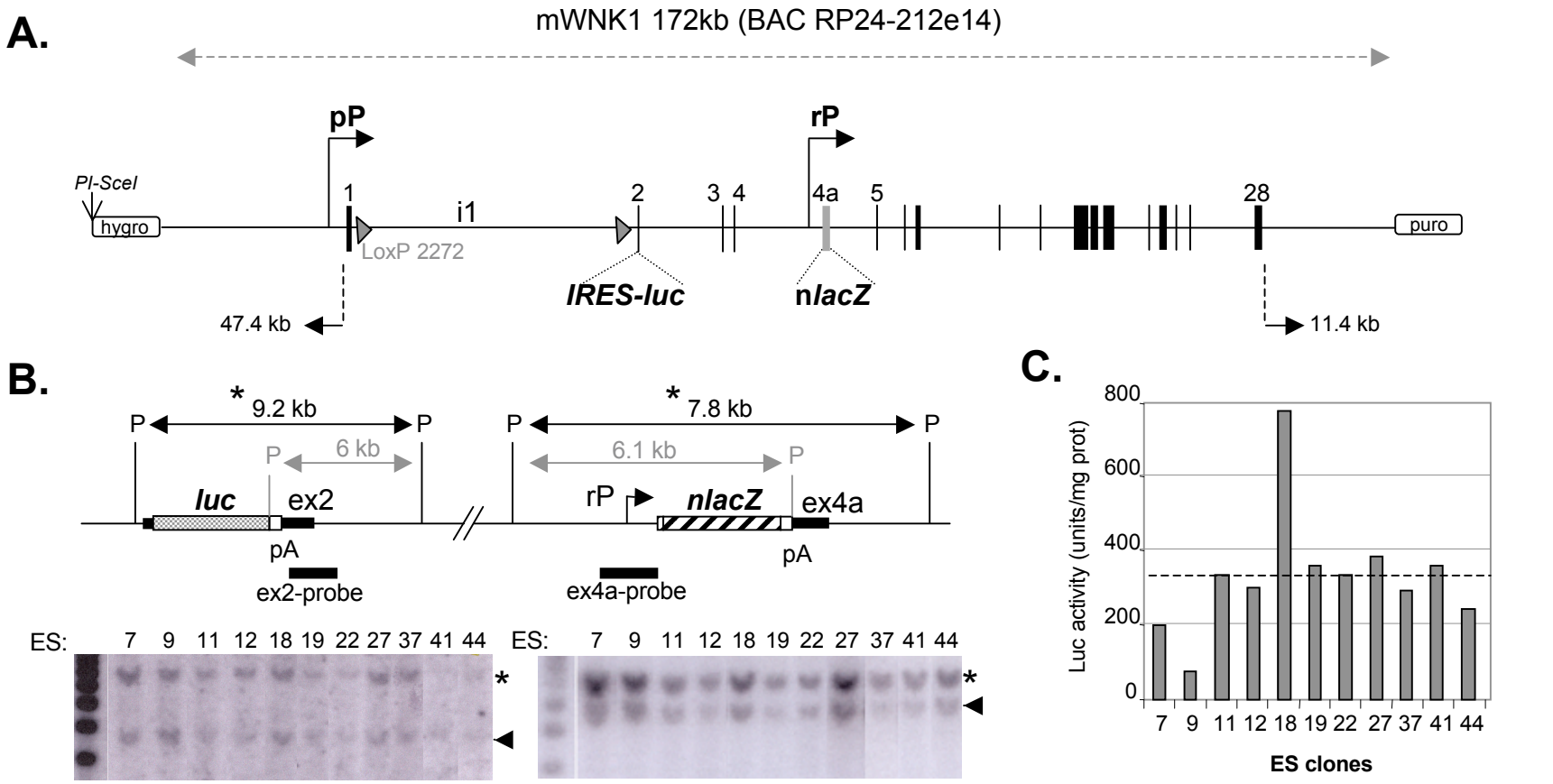

Figure S1. Strategy for studying normal and abnormal (after the deletion of intron 1) *L*- and *KS-WNK1* expression in a transgenic model. A: Schematic diagram of the Tgi1 transgene. The sizes of genomic sequences included in the BAC, 5' and 3' of *WNK1*, are indicated. Exons are indicated by numbered vertical lines. The KS-WNK1-specific exon (exon 4a) is represented by a grey vertical line. The two alternative promoters (pP and rP) are shown (bent arrows). The BAC was modified by inserting the IRES-luc reporter cassette into exon 2 and the nuclear *lacZ* gene (*nlacZ*) at the ATG of exon 4a. Two LoxP 2272 sites (grey triangles) were inserted just downstream from exon 1 and upstream from exon 2, for the deletion of the intron 1 sequence by *in vivo* Cre-mediated recombination. Two positive selection cassettes were inserted into the backbone of the BAC such that digestion with *PI-Sce*I gave a linear fragment flanked at each end by the pgk-hygro and pgk-puro cassettes. B: Schematic representation of exon 2 and exon 4a loci in the targeted BAC. pA: SV40 polyadenylation signal. Exon 2 and exon 4a modification was confirmed by Southern blotting of ES clone DNA digested with *PstI (P),* using exon 2 (ex2-probe) or 4a (ex4a-probe) as a probe.The expected size of the endogenous *WNK1* gene (asterix) and that of the transgene (arrowhead) is indicated. Results for positive clones are shown. C: Analysis of transgene expression in ES cells. Luc activity of ES protein extracts was normalized according to total protein concentration.

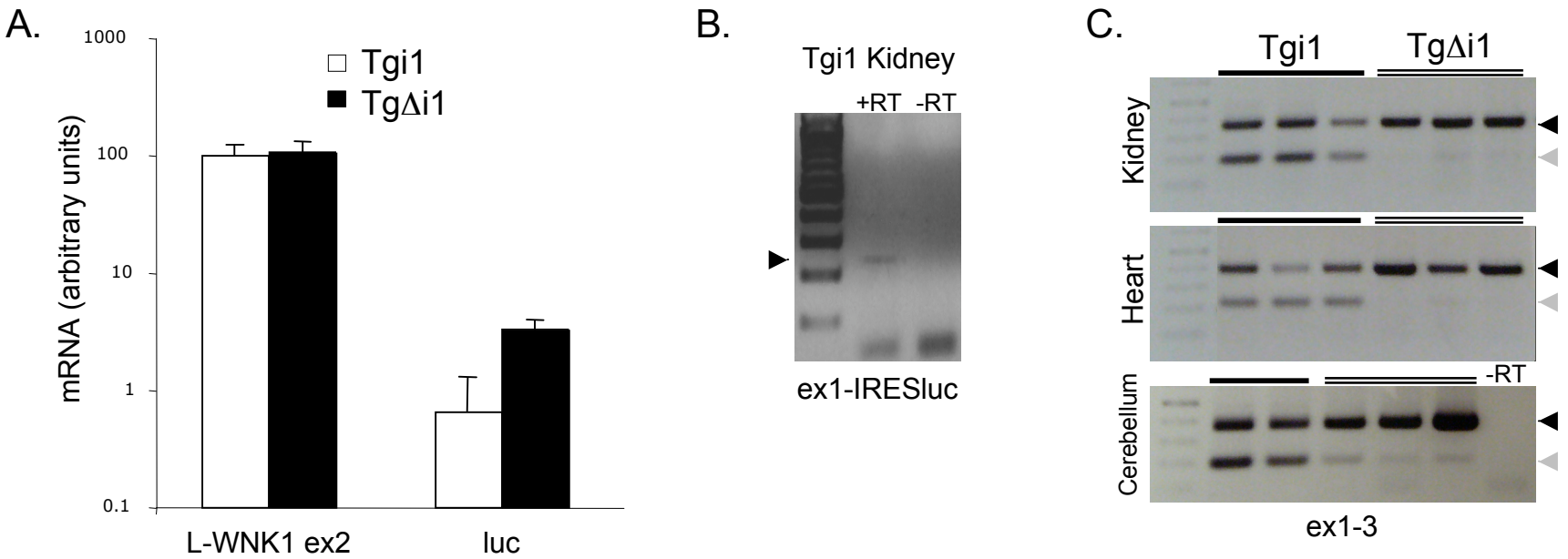

Figure S2. Transcriptional effect of intron 1 sequence. A: Relative abundance of endogenous *L-WNK1* exon 2 (L-WNK1 ex2) and luciferase (luc) mRNA in kidneys from Tgi1 and TgΔi1 mice as determined by real-time RT-PCR. Results (arbitrary units) are expressed as means + maxima from three animals (log scale). Primers specific for exon 2 are used to quantify endogenous *L-WNK1* expression and primers specific for *luc* are used to quantify luciferase expression from the transgene: *luc* is a hundred times less expressed than endogenous *L-WNK1*. B: Agarose gel electrophoresis of RT-PCR product from Tgi1 kidney RNA using forward primer in exon 1 and reverse primer in IRES-luc. The amplified fragment indicated by a black arrowhead corresponds to the splicing event between exon 1 and the modified exon 2 (ex2-IRESluc) of the BAC transgene. C: Agarose gel electrophoresis of RT-PCR products from kidney, heart and cerebellum RNAs from Tgi1 and TgΔi1 mice, with primers binding to *L-WNK1* exon 1 and exon 3. The grey arrowheads indicate the splicing event corresponding to the skipping of the transgenic exon 2-IRESluc while black arrowheads indicate the endogenous *L-WNK1* mRNA containing exon 2.

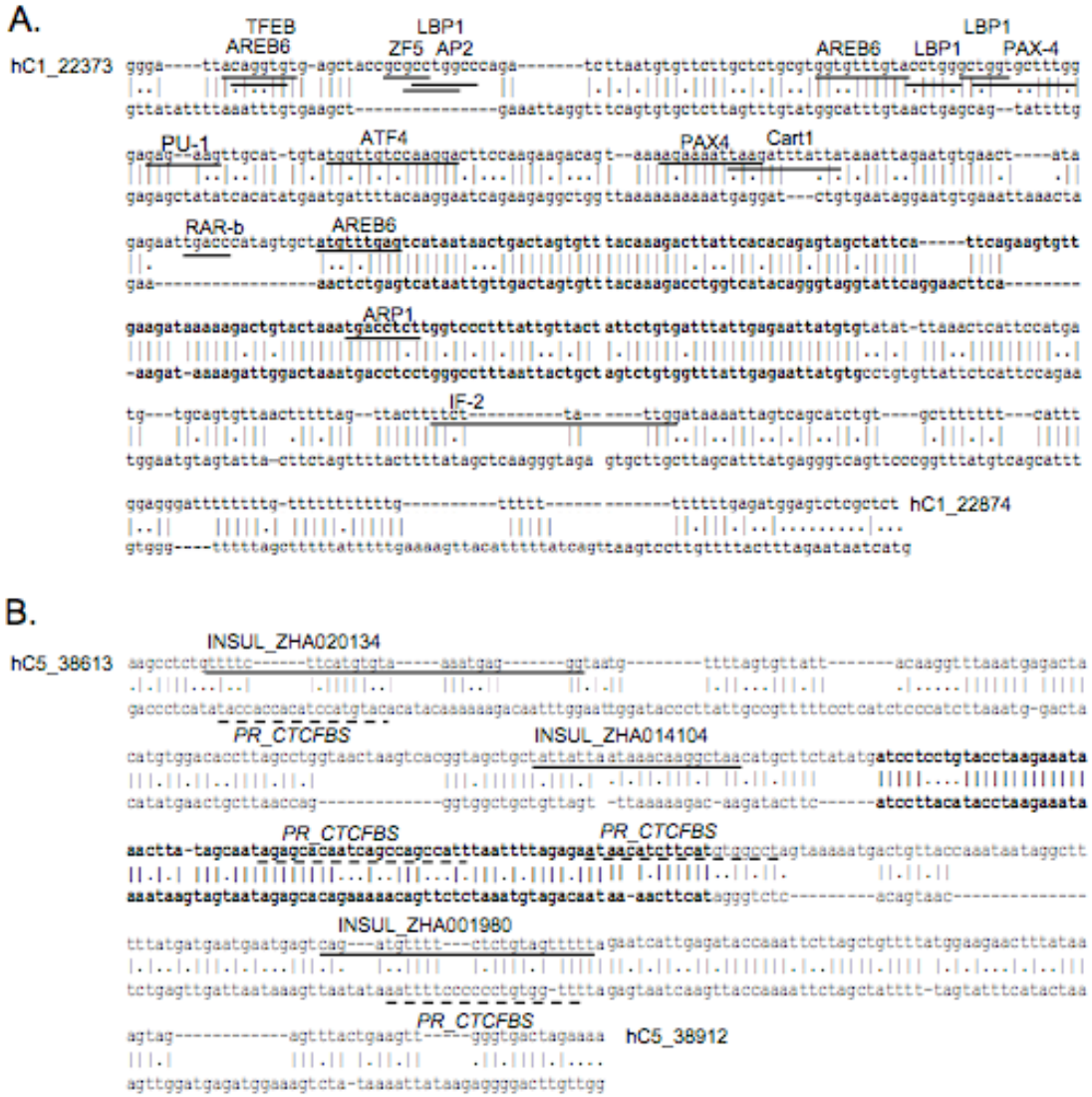

Figure S3. Comparison of human and mouse conserved non-coding sequence 1 (C1) and 5 (C5). Sequences were aligned with the EMBOSS-Align program. Sequences of the human (h) are shown in the top lines of each panel, and sequences of the mouse are shown in the bottom lines of each panel. Nucleotide position are given relative to the first nucleotide of the human intron 1. I, sequence identity; -, gaps. A. C1 sequences. Horizontal lines indicate consensus transcription factor binding sites known to act as transcriptional repressor and identified with the TESS program (0 or 1 mismatch allowed). The core sequence is in boldface. B. C5 sequences. Horizontal lines indicate sequences producing significant alignments with known insulator identified with BLASTN2.2.9 and dashed lines indicate putative CTCF binding sites identified with the CTCFBSDB prediction tool (PR_CTCFBS). The core sequence is in boldface.

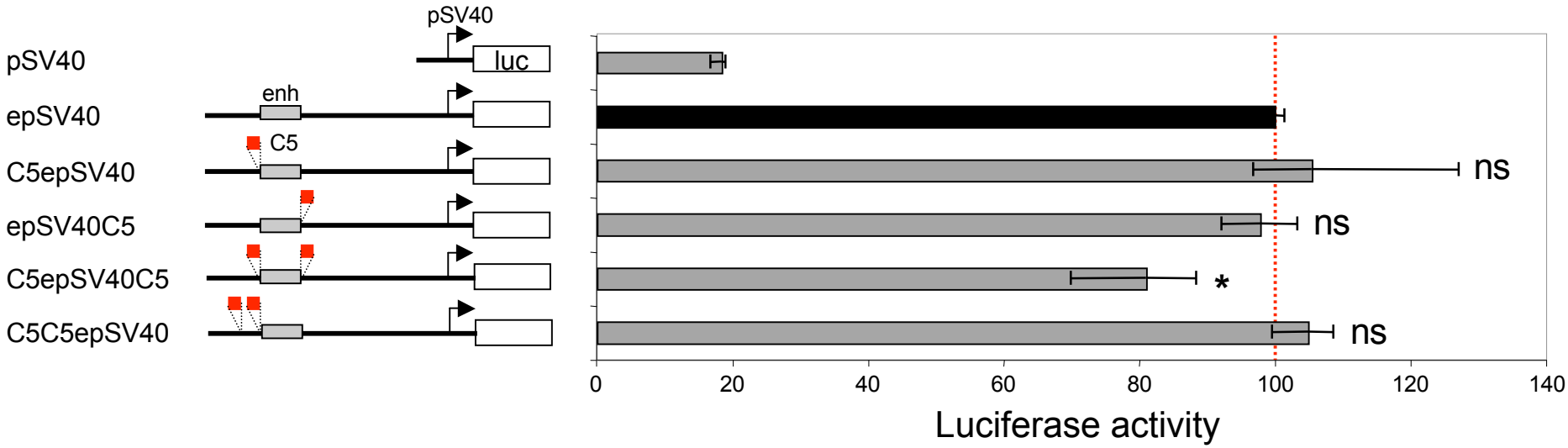

Figure S4. Enhancer blocking activity of C5. One copy of C5 (red box) was inserted on each side of the renal enhancer (enh) or between the enhancer and the SV40 promoter (pSV40). In addition, two copies of C5 were also inserted between the enhancer and pSV40 as a control. Luciferase activity was normalized with respect to that of the construct containing the enhancer and pSV40. Histograms represent means +/- minima and maxima for three experiments. ns: non-significant and *: $p<5x10^{-4}$.